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(21) International Application Number: PCT/GB91/00107 (22) International Filing Date: 25 January 1991 (25.01.91) (30) Priority data: 9001766.6 25 January 1990 (25.01.90) GB (71) Applicant (for all designated States except US): THE UNIVERSITY COURT OF THE UNIVERSITY OF GLASGOW [GB/GB]; No. 2 The Square, University Avenue, Glasgow G12 8QQ (GB). (72) Inventor; and (75) Inventor/Applicant (for US only) : SPIBEY, Norman [GB/GB]; 51 Speirs Road, Bearsden, Glasgow G61 2LZ (GB). (74) Agents: McCALLUM, William, Potter et al.; Cruikshank & Fairweather, 19 Royal Exchange Square, Glasgow G1 3AE (GB).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US. Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: VACCINES (57) Abstract A recombinant adenovirus acting as a vector for an antigen-producing gene (e.g. a rabies glycoprotein gene) comprises a CAV-2 strain modified to contain the promoter-gene sequence within the region from the SmaI site close to the end of the inverted terminal repeat up to the promoter for the early region 4 (E4). To assist replication the recombinant virus is transfected into a cell line expressing Ela proteins. The recombinant virus is used for the production of a corresponding vaccine.		

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VACCINES

The present invention relates to vaccines for use in carnivora and in particular to viral vaccines against diseases such as rabies, canine and feline parvovirus, and feline leukaemia virus. These would have distinct advantages over those in current use.

Recently there has been proposed a DNA vector system wherein a gene coding for the target antigen is inserted into a live adenovirus which is then formulated in an enteric coated dosage form.

However, many adenoviruses are known. The selection of a suitable virus to act as a vector for the gene, and the identification of a suitable non-essential region as a site for insertion of the gene pose a challenge. In particular, the insertion site must be non-essential for the viable replication of the virus and its effective operation in vivo. Moreover, the insertion site must be capable of accepting a considerable amount of new genetic material, whilst ensuring that the virus continues to replicate. The CAV-2 adenovirus is known to be a safe vector but its DNA comprises some 31,000 base pairs which must be searched to identify a suitable site or indeed to establish whether a suitable site exists or not.

The present inventors have now identified a suitable non-essential region in strains of live non-pathogenic immunogenic canine CAV-2 type adenovirus and have

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succeeded in inserting genes from pathogenic carnivora viruses with suitable expression control systems without prejudicing the stable reproducibility of the adenovirus vector.

A first aspect of the present invention provides a recombinant virus for producing antibodies or cell mediated immunity to an infectious organism in carnivora, which comprises a live non-pathogenic immunogenic viable canine adenovirus modified so as to contain a gene coding for the antigen which corresponds to said antibodies or induces said cell mediated immunity, in association with an effective promoter for said gene formed and arranged for expression of said antibodies or cell mediated immunity elements in immunogenic non-pathogenic quantities.

Preferably said canine adenovirus is a CAV-2 vaccine type strain of known long term safety modified so as to contain the inserted gene at a position close to the right hand terminus of the viral genome.

The inverted terminal repeat (ITR) is a DNA sequence found at both ends of the adenoviral genome. All adenoviruses examined to date have been shown to contain an ITR; however the lengths of these vary between serotypes. The ITR in CAV-2 is 197 base pairs (i.e. the region 0 to 197). The ITRs contain sequences which are essential for viral DNA replication and efficient packaging of the viral genomic DNA. However, we have identified a region at the end of the ITR of CAV-2 which

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can accommodate a substantial amount of extra DNA. This region extends from approximately the position of the Sma I site close to the end of the ITR (and just inside the ITR) up to the promoter for early region 4 (E4). Thus the Sma I site provides a convenient position into which foreign genes may be inserted. Other restriction sites may also be engineered into this region.

The SmaI site is a very convenient site at which to insert the gene, notwithstanding that it lies just inside the ITR. However, the insertion of material further into the ITR is unlikely to be successful as viral functions require the ITR's at the opposite ends of the virus to be able to line up together, which requires close sequence homology between the two ITR's.

The chosen non-pathogenic CAV-2 viral genome has been cloned into bacterial plasmids as a series of overlapping restriction fragments. A cloned fragment was required which spanned the Sma I site mentioned above and also included the righthand terminus of the virus. A clone carrying the 3.0 kb Sal I B fragment was therefore chosen.

Various genes may be inserted in the adenovirus DNA in accordance with the present invention to provide protection against a wide range of diseases and many such genes are already known in the art - the problem heretofore having been to provide a safe, convenient and effective vector for the genes.

Genes which may usefully be inserted include:

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- 1) Gene(s) for the capsid proteins of canine parvovirus. There is in reality only one gene. However, differential splicing results in the production of two viral capsid proteins.
- 2) The genes for the capsid proteins of feline panleukopenia, which is a parvovirus very closely related to the canine parvovirus.
- 3) Genes (or segments) coding for the peplomers of canine and feline coronavirus.
- 4) Genes for the hemagglutinin and capsid antigens of canine distemper virus.
- 5) The gene for the envelope glycoprotein of feline leukemia virus.
- 6) The gene for the envelope glycoprotein of rabies virus (various strains)
- 7) The gene for the envelope glycoprotein of feline immunodeficiency virus (FIV).

It is also possible that only fragments of genes may be used (where these are sufficient to generate an immunogenic protein or immunogenic cell response) rather than the complete sequence as found in the wild organism.

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Where available, synthetic genes may also be used. However, the present invention can be used with a wide variety of genes and is not limited to those set out above.

In some cases the gene for a particular antigen may contain a large number of introns or may be from an RNA virus, in these cases a DNA copy (cDNA) may be used.

In order for successful expression of the gene to occur, it must be inserted together with a suitable promoter sequence. Suitable promoter sequences for the genes mentioned above are known. The promoter is selected to give optimal expression of immunogenic protein or optimal cell mediated immunity according to known criteria.

The protein produced by expression in vivo in a recombinant virus-infected cell may be itself immunogenic. Moreover, the gene may produce more than one effective protein; or more than one foreign gene may be inserted in the viral genome.

Thus with the recombinant virus of the present invention, it is possible to provide protection in carnivora against a wide variety of diseases.

In a second aspect the present invention provides a method of preparing a viable recombinant virus for producing antibodies or cell mediated immunity to an infectious organism in carnivora, which comprises inserting into a live non-pathogenic canine adenovirus the gene coding for the antigen which corresponds to said antibodies or induces said cell mediated immunity,

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together with an effective promoter therefore, to produce live non-pathogenic immunogenic recombinant adenoviruses.

Generally, the promoter-gene construct is cloned into a DNA sequence which is only a part of the entire viral genome. This sequence may be present in a plasmid which allows successful cloning to produce many copies of the sequence. The cloned sequence may then be included in the complete viral genome by the use of restriction enzymes corresponding to the restriction sites at each end of the sequence.

A third aspect of the invention relates to a plasmid containing such a viral DNA sequence containing a promoter-gene construct..

The DNA sequences of the virus and the construct may be identical to the native form or may be equivalent thereto in terms of the protein expressed. Moreover, the immunological protein (and hence the DNA) may have additions, deletions or substitutions immaterial to its immunological effect.

A fourth aspect of the invention provides a vaccine formulation which comprises the recombinant vaccine in association with an acceptable carrier. The vaccine may be formulated for a administration by oral dosage (e.g. as an enteric coated tablet), by injection or otherwise.

Embodiments of the invention will now be described by way of example only. Standard methods are as described in "Molecular Cloning - A Laboratory Manual", Second Edition,

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Sambrook, J., Fritsch, E.F., and Maniatis, T., Cold Spring Harbor Laboratory Press 1989.

Example 1 (in vitro ligation)

A gene of interest such as the rabies glycoprotein gene is first put under the control of a chosen promoter, (a number of different promoters are possible). The promoter-gene construct is then cloned into the Sma 1 site of the Sal 1 B fragment contained within a plasmid. This results in a plasmid which carries the right terminal 3.0kb of CAV-2 with a chosen piece of DNA inserted just inside the boundary of the ITR.

Briefly, the modified right terminal section of the adenovirus genome is then incorporated into a complete viral genome as follows: Viral DNA is purified and cut with the restriction enzyme Sal 1, the cloned terminal Sal 1 fragment containing the promoter-gene construction is then released from the carrier plasmid and mixed with the cut viral DNA. The DNA fragments are then ligated back together.

A number of steps are taken to minimise the reformation of wild type genomes and to maximise the formation of the "correct" recombinant. For example the Sal I digested viral DNA can be run through a sucrose gradient in order to remove the right hand terminal Sal I fragment prior to ligation with the recombinant right hand terminal fragment.

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The recombinant viral DNA is reintroduced to cultured dog cells using standard procedures in order to culture the virus. Individual viral plaques are picked and expanded in order that their genomic DNAs can be examined to confirm the presence of the recombinant gene.

Ligation products have been made by this method which incorporate the rabies gene, and the feline leukemia gene.

The DNA sequence of the right hand terminal region of the canine adenovirus type 2 genome is presented in Figure 1. The sequence is shown in the conventional 5' to 3' direction numbering from the righthand terminus. For clarity only the upper strand is shown. Various features are indicated: (1) the end of the inverted terminal repeat (ITR) at position 197; (2) the Sma I restriction enzyme cleavage site at position 180-185; (3) the TATA box of the E4 transcription unit at position 395-400.

Example 2 (homologous recombination)

In Example 1 the target genes have been cloned into the SmaI site of the SalI B clone and subsequently the recombinant viruses were generated by in vitro ligation of viral DNA and plasmid fragments. In a variation of this method we have taken the recombinant SalI B clones (i.e. with the target genes inserted in the usual place) and extended them past the SalI site up to the third Kpn I from the right hand terminus (see Fig.3 of N. Spibey and H.M.A. Cavanagh, J.Gen. Virol (1989), 70, 165-172). This

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was achieved by simply ligating the KpnI B fragment (which had been previously cloned into a standard bacterial cloning vector) into the appropriately cut recombinant SalI B plasmids. Because these recombinant right-hand terminal clones extend beyond the SalI site their incorporation into intact viral DNA was carried out via homologous recombination rather than by in vitro ligation.

The generation of recombinant viruses by homologous recombination is well established, briefly our methods are as follows: Viral DNA-protein complex was prepared from purified virions using standard protocols. A sample of the DNA protein complex was digested with the restriction enzyme SalI. A recombinant plasmid comprising the right terminal portion of the CAV-2 genome, extending from approximately 74 map units (the Kpn I site) up to the terminus (100 map units), with the chosen promoter-target gene construction inserted into the SmaI site near the ITR boundary, was taken and digested with a restriction enzyme such that plasmid was linearised but not cut within the CAV or target gene sequences. The two DNA samples (viral DNA protein complex and linearised plasmid) were then mixed in a molar ratio of 1:20 and transfected into a recipient cell line whose production is described hereafter.

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Example 3 (Production of Ela Protein - Expressing Recipient Cell Line).

Primary dog kidney cell cultures and established dog kidney cell cultures (MDCK cells) were taken and transfected with a mixture of two plasmids, (1) pGRIC, which comprises the left terminal EcoRI C fragment of CAV-2 cloned into the routine cloning vector bluescript. (2) pSV2-Neo, this plasmid contains the neomycin resistance gene under control of the Sv40 virus early promoter. The purpose of these transfections was to produce cell lines which constitutively produce the canine adenovirus Ela proteins (these proteins are encoded within the EcoRI C fragment, Spibey *et al.*, Virus Research, 14, (1989) 241). We hypothesised that such a cell line would be more efficient at replicating transfected viral DNA because the El proteins are already present. The El proteins have a number of functions, however their major role can be regarded as regulatory i.e. they switch on other viral genes. Therefore if these proteins are already present then the transfected viral DNA may have a greater chance of being replicated and therefore avoid the cellular degradative processes. The plasmid conferring neomycin resistance was used as a co-selectable marker, i.e. individual clones of cells were selected on the basis of their neomycin resistance and then subsequently analysed for their production of CAV-2 Ela proteins.

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Transformed (i.e. Ela expressing) cell lines were obtained from both primary and established dog kidney cultures.

Example 4 (Transfection Method)

All transfections were carried out as follows.

Day 1 Trypsinize cells and dilute them to a concentration of $1-1.5 \times 10^5$ cells per ml. The cells are then plated at 10^6 cells per 100mm dish (or the equivalent number per 175cm^2 flask).

Day 2 Wash cells once in PBS. To each dish add 5ml of serum free media containing 20ug of DNA to be transfected and 50ul of DEAE dextran (5mg/ml stock). Ensure DNA/dextran solution is well mixed before adding to the cells.

After 4-6 hours remove DNA/dextran solution and add 5ml of serum free media containing 10% DMSO, leave for 1-2 min then remove and add complete media containing 0.1mM chloroquine diphosphate. After 4 hours replace media with fresh complete media.

Incubate cells for required period of time. This can be up to 10 days to allow for a viral infection to spread throughout the whole culture.

Neomycin resistant clones were selected using standard protocols. Briefly, after transfection cells were allowed 24 hours in normal complete media before the

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addition of neomycin (800 ug/ml final concentration). The neomycin selection was continued for 7-10 days with media changed every 2 days. Resistant clones were identified, picked and expanded in neomycin free media for 3-4 days. A further round of dilution cloning in neomycin containing media was then carried out.

All cellular incubations were carried out at 37°C.

CLAIMS

1. A recombinant adenovirus for producing antibodies or cell mediated immunity to an infectious organism in carnivora, which comprises a live non-pathogenic immunogenic viable canine adenovirus modified so as to contain a gene coding for the antigen which corresponds to said antibodies or induces said cell mediated immunity, in association with an effective promoter for said gene formed and arranged for expression of said antibodies or cell mediated immunity elements in immunogenic non-pathogenic quantities.

2. An adenovirus according to claim 1 which is a CAV-2 strain modified to contain said promoter-gene sequence.

3. An adenovirus according to any preceding claim wherein the promoter-gene sequence is introduced into a region of the viral genome which extends from the SmaI site close to the end of the inverted terminal repeat (ITR) up to the promoter for the early region 4 (E4).

4. An adenovirus according to claim 3 wherein the promoter-gene sequence is introduced into the SmaI site.

5. An adenovirus according to any preceding claim wherein the introduced gene is a gene or immunogenic gene

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fragment selected from:

- (a) gene(s) for the capsid proteins of canine parvovirus;
- (b) genes for the capsid proteins of feline panleukopenia;
- (c) genes for the peplomers of canine and feline coronavirus;
- (d) genes for the hemagglutinin and capsid antigens of canine distemper virus;
- (e) genes for the envelope glycoprotein of feline leukaemia virus;
- (f) gene for the envelope glycoprotein of rabies virus; and
- (g) gene for the envelope glycoprotein of feline immunodeficiency virus.

6. A recombinant adenovirus according to any preceding claim associated with canine adenovirus Ela proteins.

7. A method of preparing a recombinant adenovirus for producing antibodies or cell mediated immunity to an infectious organism in carnivora, which comprises modifying a live non-pathogenic immunogenic viable canine adenovirus so as to contain a gene coding for the antigen which corresponds to said antibodies or induces said cell mediated immunity, in association with an effective promoter for said gene formed and arranged for expression

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of said antibodies or cell mediated immunity elements in immunogenic non-pathogenic quantities.

8. A method according to claim 7 wherein the recombinant virus is replicated by transfection into a cell line expressing canine adenovirus Ela proteins.

9. A plasmid which contains a DNA sequence fragment from said live non-pathogenic immunogenic viable canine adenovirus and promoter-gene sequence according to any of claims 1 to 6.

10. A vaccine formulation which comprises the recombinant adenovirus according to any of claims 1 to 6 together with an acceptable carrier therefor.

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Fig 1

10	20	30	40	50	60
CATCATCAAT	AATATACAGG	ACAAAGAGGT	GTGGCTTAAA	TTTGGGCGTT	GCAAGGGGCG
70	80	90	100	110	120
GGGTCATGGG	ACGGTCAGGT	TCAGGTCACG	CCCTGGTCAG	GGTGTTCCCA	CGGGAATGTC
130	140	150	160	170	180
CAGTGACGTC	AAAGGCGTGG	TTTTACGACA	GGGCGAGTTC	CGCGGACTTT	TGGCCGGGCGC
190	200	210	220	230	240
CCGGGTTTTTT	GGGCGTTGTG	CCTATTTGTG	GTTTTGTGGT	TGACAGGGTG	ACAAGGACGT
250	260	270	280	290	300
GCTGTACTTT	TTGTGAACTT	TCCGGGCCAA	CCGCCAAAGG	GAAACTGCAC	TTAACATTTA
310	320	330	340	350	360
CCACGCGCCC	ACAATTTATG	ACTGTACTTG	GCACCACTTC	CTCAAACGCC	CCGTTATATT
370	380	390	400	410	420
CCTTTTCGTT	TTCCACACGC	CCTACTTTGA	GGACTATATA	AACGCTGTGC	TTGGCATTTC
430	440	450	460	470	480
ATCCTCATAG	CTCTCCTCTG	ACAGCCAGCC	GTCCGTGAGT	ACTATGGCAG	CTTTAGGAGT
490	500	510	520	530	540
GTCTATGGGA	GCATGTTTTT	GTCTGAGGCT	TCACAAGAGT	CTAGTGGAGA	GTGTGTGTGC
550	560	570	580	590	600
TCAACTGAGA	CTTACGAACT	TTTTGCCTTC	TGAACTCGCC	GTGTGGTGTT	TAGCCTTATT
610	620	630	640	650	
AGGGCCCAGG	AAGTGCGTCC	GTGTCCTACC	TTGCCGCGGC	TGTTTCGGTT	TA